

## Transglycosidases and Fungal Cell Wall $\beta$ -(1,3)-Glucan Branching

Vishukumar Aimanianda\*

Unité des *Aspergillus*, Institut Pasteur, 25 rue du Docteur Roux, Paris 75015, France

### Abstract

Fungal cells are endowed with a cell wall that plays a crucial role in the fungal life, by providing mechanical strength and protecting fungal cells from their environment. Chemically, this fungal cell wall consists of different polysaccharides, contributing to 80-95% of the cell wall dry mass. The core cell wall structure is made up of  $\beta$ -(1,6)-branched  $\beta$ -(1,3)-glucan linked to chitin, and is common to all fungal species. Branching leads to  $\beta$ -(1,3)-glucan ramification, facilitating its cross-linking with chitin as well as other cell wall components resulting in the construction of a functional fungal cell wall. Recently, using *Saccharomyces cerevisiae* as the model, we showed that the dual activity associated with CAZy family GH72 transglycosidases (<http://www.cazy.org/>) belonging to the GAS-family, are capable of elongating as well as branching  $\beta$ -(1,3)-glucan, an essential phenomenon during cell wall biogenesis and remodeling. Not only GAS-proteins, but also GEL-family protein from *Aspergillus fumigatus* (a pathogenic fungus) showed  $\beta$ -(1,3)-glucan elongating-branching activity. Interestingly, this dual activity was shown by only those GH72 family members containing a Carbohydrate Binding Motif-43 (CBM), suggesting that branching activity is universal in the fungal kingdom. Disruption of  $\beta$ -(1,3)-glucan branching resulted either in a sick-phenotype or led to inviability, suggesting that  $\beta$ -(1,3)-glucan branching is an essential phenomenon during fungal cell wall biogenesis. In this commentary, future perspectives on our findings are presented.

### B-(1,3)-Glucan Branching is Essential During Fungal Cell Wall Construction

GAS and GEL-proteins from *S. cerevisiae* and *A. fumigatus* belong to multigene families, containing five and seven members, respectively. Of the GAS-proteins (Gas1-Gas5), Gas1 and Gas2 are with a CBM; however, during vegetative growth, only GAS1 and GAS5 are highly expressed [1,2]. Whereas, of the seven GEL-proteins (Gel1-Gel7), Gel1, Gel2 and Gel4 are constitutively expressed during mycelial growth and of them, only Gel4 is possess a CBM [3,4]. To note here, during vegetative growth only two-three GAS/GEL-family proteins were expressed, suggesting that glucan elongation rate is higher than that of the branching, which is in accordance with the finding that  $\beta$ -glucan length is upto 1500 glucose units [5], while the branching percentage is in the range of 4-6% [1]. On the other hand, of the two-three GAS/GEL-family proteins expressed, only one contains a CBM, suggesting that there is functional redundancy of the elongating activity while branching activity is limited and associated with a unique protein. In accordance, the deletion of GEL1 or GEL2 in *A. fumigatus* resulted in a mutant without any drastic phenotypic defects [6,7], whereas GEL4 deletion was lethal [3] and the GAS1 deletion in *S. cerevisiae* resulted in a mutant with reduced viability with altered phenotype and reactivity towards cell-wall perturbing agents such as Congo Red and calcofluor white [8-10] suggesting that, though comparatively small in percentage in the cell wall, the branching of  $\beta$ -(1,3)-glucan is an essential phenomenon during fungal cell wall construction.

### Branching Activity is Dependent on the Presence of a CBM in the Gas/Gel Protein Sequence

Sequential elongation followed by branching of  $\beta$ -(1,3)-glucan and the requirement of a CBM for this dual activity associated with GAS/GEL-protein are noteworthy. Without a CBM in their sequence GAS/GEL-proteins showed only elongating activity, which suggests that CBM is essential for the branching activity, but not for the  $\beta$ -(1,3)-glucan elongation, as GAS/GEL-proteins without a CBM still showed elongating activity. A prerequisite of  $\beta$ -(1,3)-glucan elongation for the subsequent branching activity suggests that there is a requirement for the minimum size of the substrate for the branching activity. Upon acquiring a defined size, these elongated  $\beta$ -(1,3)-glucan oligomers will be possibly held by CBM in an orientation that favors the introduction of  $\beta$ -(1,6)-branching. The observation that CBM43 module non-

catalytically binds to laminarin [10,11] supports this hypothesis ours. Yet another argument could be that elongated substrate modify micro-environment facilitating the binding of the elongated  $\beta$ -(1,3)-glucan oligomer by a CBM for further branching. Therefore, a CBM is essential for proper positioning of the substrate for branching activity. We attempted to delete the entire CBM from the *S. cerevisiae* Gas1 protein; however, the truncated protein lost its both elongating and branching activity, plausibly due to the fact that CBM spans 90-100 amino acid residues in the C-terminus of Gas1 protein and its deletion may affect the functional protein structure. Alternative approaches, such as *in vitro* studies involving point-mutation in the CBM region and close monitoring of the reaction environment may be useful in understanding the mechanism of  $\beta$ -(1,3)-glucan branching and we are currently working on this aspects.

### B-(1,3)-Glucan Biosynthesis and Remodeling are Sequential Processes

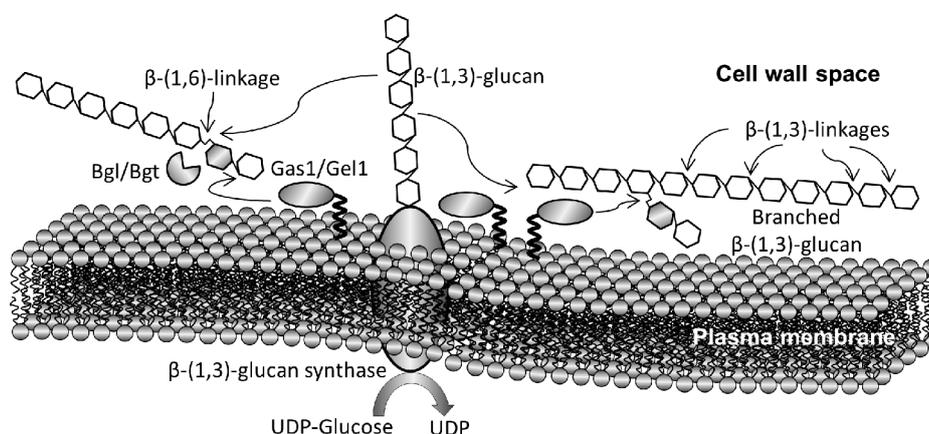
Fungal cell wall construction is an extracellular phenomenon;  $\beta$ -(1,3)-glucan biosynthesis takes place at the plasma membrane by membrane bound synthases encoded by FKS genes [12,13], which extrudes neosynthesized  $\beta$ -(1,3)-glucan chains into the cell wall space wherein it undergoes further modification. GAS/GEL-proteins are putatively glycosylphatidylinositol (GPI) anchored [2,3] and it is interesting to note that most of these genes are located near to  $\beta$ -(1,3)-glucan synthase genes (FKS); for example, ScFKS1 and ScGAS2 on chromosome XII, ScFKS3 and ScGAS1 on chromosome XII (<http://www.yeastgenome.org/>) and AfFKS1 and AfGEL7 on chromosome VI (although GEL7 is not expressed during growth) while AfGEL2 is upstream of AfFKS1 (<http://www.aspergillusgenome.org/>). In fact,

\*Corresponding author: Vishukumar Aimanianda, Unité des *Aspergillus*, Institut Pasteur, 25 rue du Docteur Roux, Paris 75015, France, Tel: 33145688225; E-mail: [vkumar@pasteur.fr](mailto:vkumar@pasteur.fr)

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**Figure 1:** A hypothetical model showing the sequence of  $\beta$ -(1,3)-glucan branching in the fungal cell wall.

In brief, short  $\beta$ -(1,3)-glucan chains synthesized by the plasma membrane bound  $\beta$ -(1,3)-glucan synthases (FKS protein) and extruded into the cell wall space will be taken-up by GPI-anchored GAS/GEL proteins facing the cell wall space and are elongated. Following, GAS/GEL proteins introduce  $\beta$ -(1,6)-linkages on adequately elongated  $\beta$ -(1,3)-glucan. On the other hand, linear  $\beta$ -(1,6)-linkages are introduced on short neosynthesized  $\beta$ -(1,3)-glucan by BGL/BGT proteins, which are further elongated and branched by GAS/GEL proteins. Plausibly FKS, GAS/GEL and BGL/BGT proteins are located nearby, facilitating  $\beta$ -(1,3)-glucan elongation and branching in the cell wall space

proteomic analyses of the *A. fumigatus* membrane fraction showed the presence of both Fks and Gel proteins and fluorescent tagged Gas1 was found to be localized in the plasma membrane [14,15], suggesting that GAS/GEL proteins are ready to act on the  $\beta$ -(1,3)-glucan synthesized and extruded by the plasma membrane bound FKS-proteins into the cell wall. However, data supporting co-localization of both FKS and GAS/GEL in the plasma membrane are lacking. Plausibly fluorescent tagging of both FKS and GAS/GEL proteins may be useful, as co-precipitation studies may not be feasible as due to  $\beta$ -(1,3)-glucan synthase, which is a membrane protein harboring multipass transmembrane domains [13,16] that lowers the ease of this protein solubility. Nevertheless, possible localization and mechanism of  $\beta$ -(1,3)-glucan branching is depicted in Figure 1.

### Biological Relevance of the Dual Activity of the Enzyme

The dual activity of enzymes is quite well documented. A few to mention, tyrosine hydroxylase from *Toxoplasma gondii* can utilize either phenylalanine or tyrosine to generate L-DOPA [17], 3 $\beta$ ,20 $\alpha$ -hydroxy steroid oxidoreductase from calf fetal RBC [18], a novel plant enzyme showing dipeptidyl peptidase III and an atypical Nudix hydrolase activities cleaving peptide and phosphate linkages, respectively [19], peroxiredoxin possessing peroxidase and catalase activity [20], neopullulanase showing hydrolysis and transglycosylation at  $\alpha$ -(1,4)- and  $\alpha$ -(1,6)-glucosidic linkages [21] and AmiA in Chlamydia with amidase and carboxypeptidase activity [22]. But our current findings along with these examples raise the question, what is the biological relevance of such dual activity? The dual function of AmiA is attributed to its peptidoglycan remodeling activity and modulating immune reactivity of peptidoglycan [22] and tyrosine hydroxylase for specific metabolic requirement to perform different biological roles during each life cycle stage [17]. Neopullulanase from *Bacillus subtilis* is a single enzyme with combined functions of  $\alpha$ -amylase, pullulanase, cyclomaltodextrin glucanotransferase and  $\alpha$ -D-(1,4)-glucan branching enzyme [21]. The activity of GAS/GEL proteins with a CBM could be compared to neopullulanase; showing  $\beta$ -(1,3)-hydrolase,  $\beta$ -(1,3)-elongase and  $\beta$ -(1,6)-branching activity. Currently we do not have explanation for such a dual activity shown by GAS/GEL proteins; however, it is tempting to speculated that the cell wall

biogenesis/modification being extracellular phenomenon, fungus may try to minimize the utilization of energy in producing enzymes for each different function and transporting them to their site of action, i.e., the cell wall.

### $\beta$ -(1,3)-Glucan Branching Machineries as the Potential Antifungal Target

To conclude, there is an urge for new anti-fungal drugs and identification of novel antifungal targets as most of the fungal pathogens are acquiring resistance towards currently available antifungals. Of the currently in-use antifungals, those targeting fungal cell wall biosynthesis, the echinocandins, have been proved to be the best in clinical use [23,24], suggesting that the fungal cell wall is an ideal antifungal target. Our study has identified a potential antifungal target, i.e., proteins involved in the  $\beta$ -(1,3)-glucan branching, as especially in case of *A. fumigatus*, GEL4-deletion was found to be lethal while Gel4 protein showed dual  $\beta$ -(1,3)-glucan elongating-branching activity. Enzyme inhibitors are commonly their substrate analogues, as competitive inhibitors. It should be borne in mind in case of GAS/GEL proteins that their substrates are long  $\beta$ -(1,3)-glucan oligomers; in *in vitro* studies, they require  $\beta$ -(1,3)-glucan oligomers containing minimum eleven glucose units [25]. Therefore, it will be difficult to develop a substrate analogue that will inhibit GAS/GEL function by acting on these enzyme active sites, such as inhibitors will be posed with the problem of traversing the cell wall, reaching their target. Alternative approaches could be either non-competitive inhibition of the enzyme activity or depleting the availability of the substrate for the GAS/GEL activity and currently we are working on these perspectives.

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